Effects of tetracycline on wild-type and inducible
P35So IPT-5/TETR transgenic tobacco plants

Lieve Quantena, Laury Chaerleb, Jean-Paul Nobenc, Harry Van Onckelen4, Els Prinsene,
Dominique Van Der Straetenc and Roland Valckeab,*

aLaboratory of Molecular and Physical Plant Physiology, Department SBG, Centre for Environmental Sciences, Building D, Hasselt University, Agoralaan, B-3590 Diepenbeek, Belgium
bUnit Plant Hormone Signalling and Bio-imaging (HSB), Department of Molecular Genetics, Ghent University, K. L. Ledeganckstraat 35, B-9000 Gent, Belgium
cLaboratory of Immunology – Biochemistry, Building A, Hasselt University, Biomedisch Onderzoeksinstituut, Agoralaan, B-3590 Diepenbeek, Belgium
4Laboratory of Plant Biochemistry and Physiology, University of Antwerp, Middelheimcampus G.U513, Groenenborgerlaan 171, B-2020 Antwerpen, Belgium
*Laboratory of Plant Biochemistry and Physiology, University of Antwerp, Middelheimcampus G.U515, Groenenborgerlaan 171, B-2020 Antwerpen, Belgium

Correspondence
*Corresponding author,
e-mail: roland.valcke@uhasselt.be

Received 12 December 2006; revised 12 January 2007
doi: 10.1111/j.1399-3054.2007.00888.x

Introduction of the Agrobacterium ipt gene, coding for isopentenyl transferase, under control of a tetracycline (Tc)-inducible promoter results in a very specific system in which cytokinin levels can be changed. Because Tc belongs to the group of antibiotics that affect 70S ribosomes, it is important to study the effects of Tc on untransformed plants. Although 1 mg l−1 Tc was previously reported to have no physiological effects, this study revealed several changes in hydroponically grown wild-type Nicotiana tabacum L. cv. Wisconsin. Therefore, lower Tc concentrations (0.1 and 0.2 mg l−1 Tc) were used to induce ipt-transgenic (tr) plants. Upon induction, real-time PCR analysis showed that the ipt gene was expressed several times higher in roots of tr plants, but not in leaves. Consequently, cytokinin levels were also elevated to a large extent in roots. This resulted in a disturbance of the cytokinin to auxin ratio, leading to an obstructed root growth. In leaves, no significant increase in cytokinins was observed. However, phenotypic and physiological effects, which could be attributed to cytokinin, were apparent in leaves of ipt-induced trs: chlorophyll and carotenoid content were elevated and grana stacking increased. Our study demonstrates that caution has to be taken to determine the ‘safe’ concentration of inducers when using inducible gene-expression systems.

Introduction

One of the main goals of plant biologists is to gain insight into the relation between structure and function in higher plants. To investigate complex processes such as development, signal transduction and photosynthesis, model organisms (e.g. Arabidopsis thaliana, rice), a diversity of mutants, and transgenics (trs) with a higher or lower expression level of a gene of interest are widely used. Several systems have been designed to overexpress a transgene. As a first approach, constitutive promoter-driven

Abbreviations – ACC, 1-aminocyclopropane-1-carboxylic acid; CK, cytokinin; Ct, cycle threshold; DZRMP, dihydrozeatin riboside-5′-monophosphate; EM, electron microscopy; IP, N6-(Δ2-isopentenyl) adenine; iPRMP, N6-(Δ2-isopentenyl) adenosine-5′-monophosphate; ipt, isopentenyl transferase; RT-PCR, real-time polymerase chain reaction; Tc, tetracycline; TetR, Tet repressor; tr, transgenic plant; wt, wild-type; Z, zeatin; Z9G, zeatin-9-glucoside; ZR, zeatin riboside; ZRMP, zeatin riboside-5′-monophosphate.
systems can result in the major enhancement of expression levels, but do not allow temporal control (Abel et al. 2004, De Block et al. 1987, Vaeck et al. 1987). On the other hand, inducible plant promoters controlled, for example, by light (Beinsberger et al. 1991), heat shock (Rupp et al. 1999, Van Loven et al. 1993) or wounding (Firek et al. 1993) offer the possibility to regulate transgene expression. As a drawback, these systems use plant-specific promoters and therefore, induction of the transgene will concomitantly affect the expression pattern of a series of endogenous genes. This lack of specificity can result in dramatic physiological changes in the phenotype of the tr plant. A gene-expression system switched on or off by a specific chemical substance, without any effect on other plant processes, is highly desirable. The use of control elements from other organisms, that respond to signals not encountered in plants, led to the development of specific inducible systems. One such system is based on the Tet repressor (TetR) of Escherichia coli (Gatz and Quail 1988, Gatz et al. 1992, Röder et al. 1994). Transgenic plants constitutively synthesizing TetR were transformed with the gene of interest under control of the ‘Triple Op’ promoter. This is a modified CaMV 35S promoter containing three operator sequences in the vicinity of the TATA box (Gatz et al. 1992). The constitutively synthesized TetR binds to the operator sequences, leading up to 100-fold repression. Adding tetracycline (Tc) results in the formation of the TetR–Tc complex, preventing the repressor TetR from binding to its operator sequences. Consequently, the gene of interest will be expressed. Because of the high association constant of the TetR–Tc complex (Gatz and Quail 1988, Hillen et al. 1983), low concentrations of Tc are sufficient to induce expression, and Tc can be applied through roots (Gatz et al. 1992). Like other antibiotics, Tc diffuses readily through membranes and is therefore likely taken up efficiently by plant cells. Because Tc belongs to the group of antibiotics that affect 70S ribosomes, effects of Tc on protein synthesis may be expected in chloroplasts and mitochondria, at concentrations reported in the literature. It is therefore important to use Tc at concentrations that do not inhibit chloroplast and mitochondrial protein synthesis, but that still induce the transgene in an efficient way.

Cytokinins have clearly been shown to play a critical role in plant growth and differentiation (Mok 1994). Cytokinin levels can be manipulated by introducing the Agrobacterium tumefaciens ipt gene, coding for an isopenetyl transferase, under control of the Tc-inducible promoter. In this study, we describe the effects of Tc on wild-type (wt) plants, the functioning of the P35So IPT-5/TETR-construct, the effects of ipt induction and the consequently increased cytokinin levels in tr plants. Although it was stated that 1 mg l⁻¹ Tc in hydroponic culture has no physiological effect on wt tobacco plants (Gatz 1995, Gatz et al. 1992, Petracek et al. 1998, Zeidler et al. 1996), we observed significant changes in morphology, pigment content and chloroplast ultrastructure at electron microscopic level in wt plants treated with this concentration of Tc. Hence, this study reveals that applicability of this system requires the use of lower Tc concentrations.

Materials and methods

Cultivation of plants

Wild-type and tr plants (Nicotiana tabacum L. cv. Wisconsin) were cultivated in a greenhouse. The tr plants were transformed by Faiss et al. (1997) and contain the ipt gene of A. tumefaciens under control of the Tc-inducible promoter (P35So IPT-5/TETR) designed by Gatz et al. (1992). Additional illumination was provided 16 h a day with AgroSon T (400 W) and HTQ (400 W) lamps [photon flux density of 200 μmol quanta (m⁻² s⁻¹)]. After sowing in potting soil, 2-week-old seedlings were put on grodan™ (Grodan A/S, Hedehusene, Denmark) saturated with half-strength Hoagland solution. After 2 weeks, plants were transferred to hydroponic culture boxes containing 2-l aerated full strength Hoagland solution, which was changed every other day. Three plants were put together in one box. After 1 week of acclimation, plants were treated with Tc (in a range of 0.1–2 mg l⁻¹ Tc). Also this solution was changed every other day. Plant shoot area was determined by time-lapse imaging using a robotized imaging system.

Electron microscopy and quantification of the photosynthetic pigments

For electron microscopic studies, parts of leaf tissue were fixed at 4°C overnight in 2% (v/v) glutaraldehyde in 50-MM sodium PIPES, pH 7.5 and post-fixed in 2% (w/v) OsO₄ in 0.2 M sodium cacodylate buffer for 2 h. After dehydration, the fixed tissues were embedded in Spurr resin. Sections were stained with uranyl acetate and lead citrate and examined using a Philips EM 208S transmission electron microscope.

The quantification of photosynthetic pigments was done according to Lichtenenthal and Wellburn (1983), by measuring absorption at wavelengths 663, 646 and 470 nm in acetone (80%) extracts.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from leaf and root with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), using
a DNase step (RNase-Free DNase set; Qiagen). cDNA synthesis was performed with the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA), starting from 1 μg of total RNA. The reaction mix contained 1× TaqMan RT buffer, 5.5 mM MgCl₂, 500 μM dNTPs, 2.5 μM random hexamers, 20 U RNase Inhibitor and 62.5 U Multiscribe RT in a total reaction volume of 50 μl. The thermal profile for cDNA synthesis carried out by the iCycler Thermal Cycler (Biorad, Hercules, CA) was: 10 min 25°C, 30 min 48°C and 5 min 95°C. One RNA sample of each preparation was processed without Multiscribe RT to provide a ‘no-RT’ control in subsequent real-time polymerase chain reaction (RT-PCR) reactions. Target-specific primers were designed using the default parameters of the Primer-Express Primer/Probe design 1.0 software (Applied Biosystems), optimized primer concentrations (200 nM for ipt primers and 300 nM for 18S rRNA primers) and 5 μl of cDNA. The thermal profile using the ABI Prism 7000 Sequence Detection System 1.0 (Applied Biosystems) was 50°C for 2 min and 95°C for 10 min, followed by 40 thermal cycles of 95°C for 15 s and 60°C for 1 min. Each sample was run in duplicate and each gene assay included a ‘no-template’ and ‘no-RT’ control. After each RT-PCR run, a melting curve analysis was performed to guarantee specificity in each reaction tube (absence of primer dimer and other non-specific products). The threshold cycle (Ct) values were exported to Excel (Microsoft) for further analysis. The ΔCt values (Ct value of the ipt-amplification plot normalized with the housekeeping gene 18S rRNA; Livak 1997) were statistically analyzed. The relative quantity of the ipt expression was calculated using the comparative Ct method (ΔΔCt method; Livak 1997). This method was valid for our results because PCR efficiencies of both ipt and 18S rRNA were equally high (95%).

Extraction and quantification of cytokinins

The extraction procedure of cytokinins is based on the protocol of Redig et al. (1996). Frozen samples were ground in liquid nitrogen and transferred in Bieseleki solution (Bieseleki 1964) for overnight extraction at −20°C. Deuterated cytokinins ([6,6-D₂]IZ, [6,6-D₂]TZR, [6,6-D₂]ZNG, [6,6-D₂]ZOG, [6,6-D₂]ZROG, [6,6-D₂]IP, [6,6-D₂]IPA, [6,6-D₂]IPG, [6,6-D₂]NZNT, [6,6-D₂]IPMP; OlChemIm Ltd., Olomouc, Czech Republic) were added as internal tracers. The pellet was resuspended for 1 h at 4°C in 80% methanol and centrifuged once more. The supernatants of both fractions were pooled and dried. After dissolving in water, cytokinins were purified by a combination of solid-phase and immunoaffinity chromatography as described by Redig et al. (1996). These methods separated cytokinins into three fractions: fraction 1: free bases, ribosides and N₇-glycosides; fraction 2: ribotides; and fraction 3: N₇- and O-glycosides. Cytokinins were quantified by micro-liquid chromatography-positive electrospray-tandem mass spectrometry (μLC-MS-MS; ThermoElectron, Waltham, MA) as described previously (Prinsen et al. 1998), with minor modifications. In brief, samples (100 μl) were loaded on a Prodigy ODS (3) guard column (Phenomenex Inc., Torrence, CA) (30 mm × 1 mm; Bester, Amsterdam, The Netherlands) in MeOH-0.01 M NH₄OAc, pH 7 (5:95, v:v) for 3 min and analyzed using a Prodigy ODS (3) (50 mm × 1 mm) analytical column using a 2-min linear gradient (slope: 42.5% methanol in 0.01 M NH₄OAc, pH 7 per minute). The effluent (100 μl min⁻¹) was monitored online using the selected reaction-monitoring mode with the compound specific transitions as listed in Witters et al. (1999). Nitrogen sheath gas flow was 60 (LCQ relative units) and the heated capillary was set at 200°C. Source and capillary voltage were 4 and 10 V, respectively. The instrument was tuned with zeatin riboside (ZR) (10⁻³ M). Quantitative analysis of cytokinins was carried out by the internal standard ratio method using deuterated isotopes. Concentrations were calculated following the principles of isotope dilution and expressed in picomols per gram FW. A signal to noise ratio of 2:1 was considered to be the detection limit (Witters et al. 1999). Cytokinins of fraction 3, containing the O- and N₇-glycoside derivatives, could not be quantified because this fraction contained impurities that obstructed the chromatography columns.

NICI GC-MS analysis of ACC

ACC was extracted by a solid-phase extraction procedure (Smets et al. 2003) using d₄-ACC (400 pmol; Sigma, St Louis, MO) as internal standard. ACC conjugates were analyzed in half of the extract after dry acid hydrolysis, following Chauvaux et al. (1993). After dry acid hydrolysis ACC-C, samples were treated as free ACC. After derivatization with pentafluorobenzyl (PFB) bromide (Netting and Milborrow 1988), ACC was measured as PFB-bis-ACC in a single gas chromatography-mass spectrometry run using a negative ion chemical ionization mode (NICI GC-MS) (NH₄⁺; HP 5890 series II coupled to a quadrupole mass spectrometer (Trio 2000; WATERS-Micromass, Manchester, UK); column: 15 m BD-XLB, 0.25 mm id, 0.25 μm film diameter (J1W Physiol. Plant. 130, 2007)
Scientific, Folsom, CA), gas phase He, 120°C to 240°C; 15th min; T inj. 250°C). The diagnostic ions used are listed in Smets et al. (2003). Data are expressed in picomols per gram FW.

Experimental setup and statistical analysis

To investigate the effect of Tc on wt N. tabacum, plants were treated with respectively 0.2, 0.5, 1 or 2 mg Tc per liter Hoagland solution and compared with untreated plants. Each treatment was applied to six boxes each containing three plants. Two weeks after Tc treatment, plants were harvested and root length was measured. From the largest leaf, samples were taken for pigment quantification (all plants), and electron microscopy (three representative plants of each treatment). To take into account the possible nested box effect (three plants were put together in the same box), a model was fitted to the data of pigment quantification and root length using the statistical program SAS (proc GLM; SAS Institute Inc., Cary, NC). If the residuals were normally distributed with equal variance, multiple comparisons between the different treatments (no Tc, 0.2 mg l⁻¹ Tc, 0.5 mg l⁻¹ Tc, 1 mg l⁻¹ Tc and 2 mg l⁻¹ Tc) were carried out using the Tukey test. The level of significance α was set to 0.05.

To investigate the effects of Tc induction of the ipt gene in tr tobacco plants, tr plants were treated with two concentrations of Tc (0.1 and 0.2 mg l⁻¹ Tc). They were compared with wt treated with the same respective concentration and with untreated tr plants. Each treatment was applied to four boxes each containing three plants. In total, 36 wt plants and 36 tr plants were used. After 10 days of Tc treatment, plants were harvested and root length was measured. Samples were taken for pigment (all 12 plants), ACC (leaf and roots of nine plants) and cytokinin (leaf, stem and roots of six plants) quantification, RNA isolation (roots and leaf of six plants) and electron microscopic sections (leaves). For morphology, pigment content and ipt expression in leaves and roots, a model was fitted using the statistical program SAS (proc GLM), taking into account the possible nested box effect. If the residuals were normally distributed with equal variance, the Tukey test was used for multiple comparisons. The level of significance α was 0.05. Because the data of ACC quantification were not normally distributed, non-parametric analyses were carried out. First a Kruskal–Wallis test for independent samples was applied on the data (using StatXACT, Version 3.1, Statcon, Wittenhausen, Germany). If the result was significant (P < 0.05), a non-parametric multiple comparison procedure was carried out (Conover 1999). Because a lot of the results from cytokinin quantification were either below detection limit or could not be quantified, no further statistical analysis was applied on those data.

Results

Effect of Tc on wt and ipt-tr tobacco plant phenotype

Tc treatment of 5-week-old wt plants resulted in multiple effects: reduction of root growth, increases in leaf pigment content and changes in chloroplast ultrastructure. Fig. 1A shows the root length of wt plants treated with a range of different Tc concentrations. After 14 days of treatment with 1 mg l⁻¹ or 2 mg l⁻¹ Tc, roots of the then 7-week-old plants were significantly shorter and also appeared browner when compared with untreated plants. Subsequently, we concentrated on characterizing the lower concentrations of Tc that did not affect wt, but still had a significant effect in the ipt trs. During the treatment period, wt plants appeared as healthy as untreated tr plants. This was confirmed by unaltered kinetics in shoot growth, based on leaf area determination (data not shown). Treated tr plants on the other hand were suffering from the Tc treatment, even at the low concentrations of 0.1 and 0.2 mg l⁻¹ Tc. Severe wilting of leaves was observed, making them very difficult to handle. The root system of treated tr plants was greatly reduced and roots appeared intensely brown. Root lengths of untreated and treated (0.1 or 0.2 mg l⁻¹ Tc) wt and tr plants are shown in Fig. 1B. The length of roots

![Fig. 1. (A) Effect of Tc on root length of wt tobacco plants after 14 days of treatment. Values are means ± sd. (B) Effect of Tc on root length of wt and tr plants after 10 days of treatment. Values are means ± sd.](Physiol. Plant. 130, 2007 293)
of wt plants did not differ significantly between the three treatments ($P < 0.05$). All tr plants, including uninduced plants (tr 0), had significantly shorter roots compared with wt plants. Moreover, in the trs the length of roots shortened significantly with increasing Tc concentration.

Next, pigment content of leaves was determined upon treatment with the same range of Tc concentrations as used for the root length assays. Carotenoid content per gram FW was significantly increased when 0.5 mg l$^{-1}$ Tc or a higher concentration thereof was given to wt plants (Fig. 2A). As expected from the Fig. 2A results, carotenoid content of wt plants was not significantly influenced by Tc treatment at the concentrations of 0.1 or 0.2 mg l$^{-1}$ Tc (Fig. 2B), whereas tr plants treated with these low concentrations had a significantly higher carotenoid content ($P < 0.05$). In addition, the total chlorophyll content (chl($a+b$)) per gram FW was not significantly different for leaves of wt and untreated tr plants (Fig. 2B). Leaves of treated tr plants (tr 0.1 and tr 0.2) on the other hand had a significantly higher amount of total chlorophyll per gram FW. This increase in total chlorophyll was the highest for leaves of tr plants fed with 0.2 mg l$^{-1}$ Tc.

As a third phenotypic parameter, chloroplast ultrastructure was visualized. Upon treatment with the maximally used Tc concentration, an increase in both grana stacking and number of osmophilic droplets were observed, as compared with the wt (Fig. 3A, B). No differences in ultrastructure were seen between untreated wt and untreated trs (compare Fig. 3A with C). Induction of the ipt gene in tr plants clearly affected chloroplast ultrastructure, as shown by an increase in both grana stacking and starch accumulation (compare Fig. 3C with D).

### Tc induction of the ipt gene in tr tobacco plants

Given the above-described results, we have convincing evidence that Tc, even at concentrations that were supposedly without physiological effect (Gatz 1995), does affect plant development at the morphological, ultrastructural and biochemical levels. Because 0.5 mg l$^{-1}$ Tc still had some distinct physiological effects on wt tobacco plants, the lower concentrations 0.1 and 0.2 mg l$^{-1}$ Tc were used to induce the ipt gene in tr tobacco plants. These treatments were always applied in parallel to wt plants as an extra check for possible Tc effects.

### Expression of the ipt gene in tr plants as a function of Tc concentration

Linear ipt-amplification plots were drawn after RT-PCR analysis using cDNA produced from roots and leaves of both wt and tr plants (results not shown). As expected in the absence of ipt expression, amplification plots of wt plants did not cross the threshold. In untreated trs (tr 0), amplification plots crossed the threshold, indicating background ipt expression in both roots and leaves. In roots of trs treated with 0.1 or 0.2 mg l$^{-1}$ Tc, the relative quantity of ipt expression was respectively 5 and 18 times higher, as calculated using the ΔΔCt method with the above mentioned (tr 0) background expression level as a reference (Fig. 4). No significant increases in ipt expression were detected in leaves of treated tr plants, compared with levels in untreated trs (Fig. 4).

### Changes in cytokinin content in roots, stems and leaves of wt and tr plants as a function of Tc treatment

Table 1 presents the average cytokinin content in roots of wt and tr plants, treated with 0.2 mg l$^{-1}$ Tc (wt 0.2 and tr 0.2) or untreated (wt 0 and tr 0). Cytokinin concentrations in roots of wt plants (wt 0 and wt 0.2) and untreated trs (tr 0) were comparable to each other. Roots of treated trs (tr 0.2) contained nearly 1000-fold higher levels of zeatin riboside (ZR) and zeatin ribotide-5'-monophosphate (ZRMP), while zeatin-9-glucoside (Z9G)
and dihydrozeatin ribotide (DZRMP) increased approximately 100-fold. The other cytokinin components (iP, iPMP) did not respond to ipt induction (results not shown). Cytokinin quantification in stems and leaves of the different plant groups revealed comparable amounts for all cytokinin components (data not shown).

Changes in ACC content in roots and leaves of wt and tr plants as a function of Tc treatment

Fig. 5 displays the ACC content in roots of wt and tr plants treated with Tc (0.1 or 0.2 mg l⁻¹) or left untreated (wt 0 and tr 0). Induced trs contained significantly more ACC in their roots compared with all other plant groups. ACC did not significantly change in leaves of induced tr plants, neither were ACC conjugates increased in roots nor leaves of induced trs (results not shown).

Discussion

Uncoupling effects of Tc on the wt phenotype from specific induced cytokinin effects

Treating wt tobacco plants with 1 mg l⁻¹ Tc significantly inhibited root growth (Fig. 1A), as described previously (Böhner et al. 1999). Applying 1 mg l⁻¹ Tc to wt tomato plants also led to reduced root growth, while 0.1 mg l⁻¹ Tc had no significant effect (Corlett et al. 1996). Contrary to this, Gatz (1995) reported that rooting of hydroponically grown plants was not affected at a concentration of 1 mg l⁻¹ Tc. In our hydroponic experiments, only concentrations of 0.1 or 0.2 mg l⁻¹ Tc did not alter root growth, as shown in Fig. 1B.

Carotenoid concentration was elevated in wt plants treated with high concentrations of Tc (1–2 mg l⁻¹); the increase observed in trs at 0.2 mg ml⁻¹ Tc was specific for Tc, because effects in the wt were absent. The increase in carotenoid content (Fig. 2A, B) in wt plants treated with Tc might be linked to the observed increase in grana stacking (Fig. 3A, B). Grana contain more light-harvesting complexes (LHC) II as compared with unstacked thylakoids, and these LHC in turn contain more carotenoids in comparison with photosystems. However, this enhancement
in carotenoids could also be because of stress effects, because carotenoid pigments are associated with the protection of the photosynthetic apparatus against photodamaging effects (Garcia-Asua et al. 1998, Krinsky 1984, Siefermann-Harms 1987). Our results suggest usage of Tc at a concentration not exceeding 0.2 mg l\(^{-1}\). A concentration of 0.1 mg l\(^{-1}\) Tc is sublethal for prokaryotic cells (Geissendörfer and Hillen 1990) and therefore likely has no effect on chloroplasts or mitochondria of higher plants. It was established that Tc binds in a different way to the Tc repressor TetR than it does to the 70S ribosome (Degenkolb et al. 1991). Although the crystal structure of the TetR–Tc complex has been resolved (Hinrichs et al. 1994), synthesis of a non-antiobiotic (non-ribosome binding) inducer derived from Tc has not yet been developed.

Ipt expression in root and shoot – evidence for leakiness

Our results indicated a significant concentration-dependent induction of the ipt gene in roots of tr plants (Fig. 4). An increase in transgene induction as a function of Tc concentration was also reported by Gatz (1995). However, two unexpected results were shown when analyzing the ipt gene expression of the tr plants. First of all, RT-PCR analysis detected a background level of ipt expression in both roots and leaves of untreated tr plants, although a stringent repression in the absence of the inducer Tc was reported before (Gatz et al. 1992). As a first explanation for this finding, RT-PCR analysis as used in our study is much more sensitive compared with the generally used Northern blot analysis (Masgrau et al. 1997, Röder et al. 1994). In support of our observations, indications of incomplete repression of ipt transcription were reported in other ipt-tr plants (Faiss et al. 1997, Gan and Amasino 1995, Hewelt et al. 1994, Li et al. 1992). From a molecular viewpoint, the used TetR repressor must displace and therefore compete with at least 40 proteins that assemble around the TATA box to form a competent transcription initiation complex (Gatz 1997). Therefore, efficient repression probably depends on a constant high concentration of the intracellular repressor, suboptimal or fluctuating repressor levels could explain the observed leakiness.

As a second unexpected observation, in our study no difference in ipt expression was seen in leaves of tr plants upon Tc uptake by the roots (Fig. 4). This indicates that Tc was either not transported to the aerial parts or was inactivated before it could reach the leaves. Our results are consistent with another report describing the effects after induction of P35So IPT-5/TETR tr plants with 0.5 mg l\(^{-1}\) Tc in the hydroponic solution (Faiss et al. 1997). Contrary to our results, reports using the reporter gene (uidA) (Gatz et al. 1992) or other marker genes (Gatz 1995) under control of the Tc-inducible promoter, showed a homogeneous de-repression after Tc uptake through the roots, but importantly both studies also revealed a decrease in mRNA level of the reporter gene, 2 days after the last addition of fresh Tc. As a possible explanation, it was previously suggested that this antibiotic is light sensitive (Gatz et al. 1991). To assure constant high Tc activity, and thus ipt gene expression, the Tc solution must be changed every other day, as was done in our experiments. When Tc is applied through the roots, as was the case in our study, it has to be transported to the aerial parts of the plant to induce ipt expression in the leaves. During this time span, Tc might also be broken down.

Correlation of cytokinin levels with ipt gene expression

ZRMP, ZR and DZRMP cytokinins accumulated to significantly high levels in roots of induced tr plants (Table 1), confirming the results of other studies using ipt-transformed plants (Faiss et al. 1997, Redig et al. 1996).

### Table 1. Average (±SD) cytokinin content (pmol g\(^{-1}\) FW) in roots of different plant groups. (wt 0/tr 0: untreated wt/tr plants, wt 0.2/tr 0.2: wt/tr plants treated with 0.2 mg l\(^{-1}\) Tc).

<table>
<thead>
<tr>
<th>Cytokinin</th>
<th>wt 0 ± SD</th>
<th>wt 0.2 ± SD</th>
<th>tr 0 ± SD</th>
<th>tr 0.2 ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZR</td>
<td>2.98 ± 1.42</td>
<td>2.75 ± 1.57</td>
<td>3.02 ± 0.53</td>
<td>1057.92 ± 307.19</td>
</tr>
<tr>
<td>29G</td>
<td>3.13 ± 0.85</td>
<td>0.85 ± 0.53</td>
<td>1.71 ± 0.97</td>
<td>129.11 ± 70.54</td>
</tr>
<tr>
<td>ZRMP</td>
<td>5.09 ± 2.82</td>
<td>23.23 ± 10.88</td>
<td>11.65 ± 3.08</td>
<td>7562.81</td>
</tr>
<tr>
<td>DZRMP</td>
<td>2.75 ± 1.06</td>
<td>4.26 ± 2.82</td>
<td>2.13 ± 1.25</td>
<td>402.73</td>
</tr>
</tbody>
</table>

![Fig. 5. ACC content in roots of wt and tr plants after 10 days of treatment. Values are means ± SD.](image_url)
mediated conversion of limiting step in ethylene biosynthesis is the ACC synthase (Van Der Straeten 2005). The first and generally rate-limiting step in ethylene biosynthesis mediated conversion of S-adenosyl-Met to ACC. Cytokinins increase the stability of ACC synthase, resulting in increased ethylene biosynthesis in Arabidopsis (Chae et al. 2003). In our study, ACC levels were clearly elevated in roots of induced tr plants (Fig. 5), indicating a stress condition. Earlier observations indeed suggested that manipulating endogenous cytokinin levels led to an induction of stress-responsive genes (Crowell and Amasino 1997), leading to the induction of stress-responsive genes (Crowell and Amasino 1997), which would alter the cyt/aux ratio in a similar way as ipt overexpression. Morphology of untreated trs was comparable to wt plants, except for a decrease in root system (Fig. 1B). This might be because of the background ipt expression, and associated cytokinin production, although no significant accumulation of cytokinins was observed in roots of untreated trs (Table 1). However, a slight increase in root cytokinin levels could have been masked by the large variability in the quantitative analysis of endogenous cytokinin levels.

**How does ipt induction lead to changes in leaf pigment content?**

Cytokinins are known to lower chlorophyll degradation by reducing the chlorophyllase activity. In addition, they stimulate chloroplastic development, as well as chlorophyll synthesis and the expression of chlorophyll a/b-binding protein (Hare and Van Staden 1997, Weidhase et al. 1987). In our results, chlorophyll content increased in induced trs (Fig. 2B), although no cytokinin accumulation was observed in their leaves. Tc treatment of trs also caused an elevated carotenoid content (Fig. 2B). This increase was probably as a result of stress effects, because induced trs had an unhealthy appearance, as evidenced by severe wilting. Manipulation of endogenous cytokinin levels probably results in metabolic disturbances (Schmülling et al. 1997), leading to the induction of stress-responsive genes (Crowell and Amasino 1994). As explained above carotenoids are associated with protection of the photosynthetic apparatus against photo-oxidative damage (Krinsky 1984, Siefermann-Harms 1987). The observed increase in grana stacking in treated trs is consistent with a higher carotenoid content (Fig. 3 C, D), as explained above for wt plants treated with Tc. Moreover, cytokinins are known to stimulate the expression of LHCs (Crowell and Amasino 1994). If the increase in LHCs and total chlorophyll content of the induced trs were indeed a cytokinin effect, it could only be explained by cytokinin transport from the roots to the aerial parts, resulting in a slight increase in leaf cytokinin levels that would have been masked by the large variability in the quantitative analysis of endogenous cytokinin levels and therefore could not be proved by our results. However, the elevated level of cytokinin in roots might also induce other root-to-shoot signals that lead to an increase in chlorophyll or LHCs.

The increase in starch granules in the chloroplast might be linked with a disturbed source–sink pattern. Carbohydrates, mostly in the form of sucrose, are being loaded into the phloem at the site of the source organ and unloaded at the site of the sink organ, depending on the

---

**Plant hormone interactions and imbalances as an explanation for the phenotypic effects**

Ethylene is synthesized upon stress situations (De Paepe and Van Der Straeten 2005). The first and generally rate-limiting step in ethylene biosynthesis is the ACC synthase. Cytokinins increase the stability of ACC synthase, resulting in increased ethylene biosynthesis in Arabidopsis (Chae et al. 2003). In our study, ACC levels were clearly elevated in roots of induced tr plants (Fig. 5), indicating a stress condition. Earlier observations indeed suggested that manipulating endogenous cytokinin levels led to an induction of stress-responsive genes (Crowell and Amasino 1997), probably resulting from a metabolic disturbance (Schmülling et al. 1997), leading to the induction of stress-responsive genes (Crowell and Amasino 1997), which would alter the cyt/aux ratio in a similar way as ipt overexpression. Morphology of untreated trs was comparable to wt plants, except for a reduction of their root system (Fig. 1B). This might be because of the background ipt expression, and associated cytokinin production, although no significant accumulation of cytokinins was observed in roots of untreated trs (Table 1). However, a slight increase in root cytokinin levels could have been masked by the large variability in the quantitative analysis of endogenous cytokinin levels.
sink strength. To a large extent the reduced root system of induced tr plants likely results in a much lower sink strength compared with wt plants. Consequently, excess photosynthate produced in the leaves will be stored as starch in the chloroplasts, instead of being translocated to the roots.

In conclusion, although other reports indicate that 1 mg l⁻¹ Tc in hydroponic culture has no physiological effect on plants (Gatz et al. 1992, Gatz 1995, Petracek et al. 1998, Zeidler et al. 1996), we observed significant changes in morphology, pigment content and ultrastructure in wt plants treated with this concentration of Tc. We thus suggest using Tc at a maximum concentration of 0.2 mg l⁻¹, but even lower concentrations might be preferable. Background ipt expression is found in roots and leaves of untreated tr plants, indicating leakiness of the Tc-inducible promoter. However, the degree of leakiness was not sufficient to cause a detectable increase in cytokinin content. Upon Tc treatment, the ipt mRNA level was not elevated in leaves of tr plants, indicating that Tc either was not transported to the aerial parts of the plant or was inactivated before it could reach the leaves.

This report cautions the use of the Tc-inducible promoter overexpression system with Tc concentrations previously described in literature. In addition, several alternative systems could be considered for conditional expression of transgenes, which require very low concentrations of inducer (Tavva et al. 2006).

Acknowledgements – The authors thank Natascha Stefanie for help with Electron Microscopic preparations, Jan Daenen and Greet Clerx for technical assistance, Erik Royackers and Sevgi Oden for help with respectively cytokinin and ACC quantification and Rafaël Smets for assisting the adaptation of the LC-MS-MS procedure for cytokinin analysis. This work was supported by the Fund for Scientific Research – Belgium (F.W.O.) grant number G0068.99. L. Chaerle is a post-doctoral fellow of the Research Foundation – Flanders.

References


Rupp HM, Frank M, Werner T, Strnad M, Schmulling T (1999) Increased steady state mRNA levels of the STM and KNAT1 homeobox genes in cytokinin overproducing Arabidopsis thaliana indicate a role for cytokinins in the shoot apical meristem. Plant J 18: 557–563


